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Brain-derived human immunodeficiency virus-1 Tat exerts differential effects on LTR transactivation and neuroimmune activation

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Molecular diversity within brain-derived HIV-1 sequences is highly variable depending on the individual gene examined and the neurological status of the patient. Herein, we examined different brain-derived human immunodeficiency virus (HIV)-1 *tat* sequences in terms of their effects on LTR transactivation and host gene induction in neural cells. Astrocytic and monocytoid cells co-transfected with prototypic *tat* clones derived from non-demented (ND) ($n = 3$) and demented (HAD) ($n = 3$) AIDS patients and different HIV-LTR constructs revealed that LTR transactivation mediated by *tat* clones derived from HAD patients was decreased ($p < 0.05$). A Tat-derived peptide containing the amino acid 24–38 domain from a ND clone caused down-regulation of the LTR transactivation ($p < 0.05$) in contrast to peptides from other Tat regions derived from HAD and ND *tat* clones. Both brain-derived HAD and ND *tat* constructs were able to induce the host immune genes, MCP-1 and IL-1β. Microarray analysis revealed several host genes were selectively upregulated by a HAD-derived *tat* clone including an enzyme mediating heparan sulphate synthesis, HS3ST3B1 ($p < 0.05$), which was also found to be increased in the brains of patients with HAD. Expression of the pro-apoptotic gene, PDCD7, was reduced in cells transfected with the HAD-derived *tat* clone and moreover, this gene was also suppressed in monocytoid cells infected with a neurotropic HIV-1 strain. Thus, mutations within the HIV-1 *tat* gene may exert pathogenic effects contributing to the development of HAD, which are independent of its effects on LTR transactivation. *Journal of NeuroVirology* (2007) **13,** 173–184.

Keywords: astrocyte; chemokine; cytokine; HIV-1; LTR; macrophage; microarray; Tat

Introduction

The mechanisms underlying neurological disease caused by human immunodeficiency virus type 1 (HIV-1) remain uncertain with both pathogenic host responses and viral proteins contributing to its development (Gonzalez-Scarano and Martin-Garcia, 2005). The HIV-1 regulatory protein Tat is an 86-101 amino acid protein, which has been shown to play a pivotal role in HIV pathogenesis. Nonetheless, Tat's chief role involves regulation of HIV-1 replication (Dayton *et al*, 1986), exerting its actions by binding to an RNA transactivation response element (TAR), which folds into a hairpin structure and is located at the extreme

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5′ end of viral transcripts. In addition to binding to TAR, Tat protein can also bind and recruit several transcription factors that are required for transactivation of the HIV-1 promoter (Gatignol *et al*, 1996). Besides being necessary for HIV-1 transcription, Tat protein is also a potent modulator of cellular functions and has been shown to play an important role in the neuropathogenesis of HIV-1 infection (Lassen *et al*, 2004).

Tat-encoding RNAs have been detected in the brains of patients with HIV-associated dementia (HAD) and also in non-demented (ND) HIV/AIDS patients. (Wesselingh *et al*, 1993). Moreover, HIV-1 Tat protein is also detected in brain macrophages (Hofman *et al*, 1994) and is produced by HIV-infected astrocytes (Brack-Werner, 1999). Secreted HIV-1 Tat protein can enter and exert pathogenic effects on adjacent uninfected brain cells. Importantly, Tat activates cellular transcription factors including NF-κB, thereby increasing expression of NF-κB-regulated genes such as TNF-α, IL-1ß, and MCP-1 (Chen *et al*, 1997; Conant *et al*, 1998; Conant *et al*, 1996; Nath *et al*, 1999). These actions may have major neuropathogenic consequences, as dysregulation of cytokine and chemokine expression result in chemotaxis of leukocytes, which subsequently influence neuronal function and survival (Rappaport *et al*, 1999).

Molecular diversity within the HIV-1 genome is a key determinant of viral replication and fitness (Andreoni, 2004). Indeed, the HIV-1 Tat sequence shows substantial variation in its sequence, which appears to influence its effects on the LTR (Roof *et al*, 2002). Previous studies indicate that *tat* sequences show heterogeneity among brain-derived clones from AIDS patients (Bratanich *et al*, 1998; Mayne *et al*, 1998). While phylogenetic studies of *tat* sequences did not reveal clustering among individual clinical groups, molecular heterogeneity was greatest among patients with HIV-associated dementia (HAD) (Bratanich *et al*, 1998). Thus, Tat sequence diversity might influence viral replication or host responses, thereby contributing to the pathogenesis of HAD, as implied by earlier studies (Johnston *et al*, 2001c; Silva *et al*, 2003). Given these findings, we hypothesized that HIV-1 Tat exerted differential effects on LTR transactivation and host gene responses, which depended on the individual Tat sequence. Brain-derived HIV-1 *tat* clones from non-demented (ND) and demented (HAD) AIDS patients were studied in terms of their ability to transactivate the HIV-LTR and to elicit a host response in monocytoid and astrocytic cells, revealing that the LTR transactivation and host responses were differentially regulated by the individual *tat* clones.

Material and Methods

LTR and tat constructs

Several HIV_{IIIB}LTR-derived constructs with different truncated or deleted regions and containing firefly luciferase were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Health from Drs Josephs, Nabel, and Perkins (Gorman *et al*, 1982; Nabel and Baltimore, 1987). Brain-derived *tat* clones from HIV/AIDS patients with (HAD) or without (ND) HIV-associated dementia were constructed and sequenced, as described previously (Bratanich *et al*, 1998).

Peptide synthesis

Peptides were prepared by automated simultaneous multiple peptide synthesis (SMPS). The SMPS set-up was developed using a standard autosampler (Gilson 221) as described previously (van der Zee *et al*, 1994). Briefly, standard Fmoc chemistry with in situ PyBop/NMM activation of the amino acids in a 5-fold molar excess with respect to 2 μmol/peptide PAL-PEG-PS resin (Perseptive Biosystems) was employed. Peptides were obtained as C-terminal amides after cleavage with 90–95% TFA/scavenger cocktails. Peptides were confirmed by reversed phase HPLC and by electrospray mass spectrometry. The sequences are depicted in Figure 3A.

Cells and transfections

The human astrocytoma cell line U373 MG was cultured in DMEM/HAM F10 (v/v 1:1) supplemented with 10% FCS, 10 IU/ml penicillin, 10 IU/ml streptomycin, and 1.2 mM L-glutamine. Transfection experiments were performed using the DEAE dextran method (Aldovini and Feinberg, 1990). 100 ng of *tat* containing plasmids and 250 ng of the pHIV LTR plasmids were mixed with 1 ml of DMEM containing 125 μg of DEAE-dextran and transfected into astrocytic (U373) cells (3×10^5) by incubation for 4 hours at 37°C together with mock transfections as controls. The transfection mixture was removed and cells were incubated for 1 min with 10% dimethyl sulfoxide solution in phosphate-buffered saline (PBS). Cells were then incubated for 48 hours at 37°C before they were lysed and used in the luciferase assay. Monocytoid (THP-1) cells were cultured in DMEM, 10% FCS, 10 IU/ml penicillin, 10 IU/ml streptomycin and transfections were performed using lipofectin (Life Technologies Gaithersburg, MD) according to the manufacturer's guidelines. Monocytoid (U-937) cells stably transfected with brain-derived HIV-1 Tat clones from patients with (HAD 18 F(-83)A) or without (ND-12 L(-9)B) HAD were cultured, as previously described (Johnston *et al*, 2001a). Monocytoid (Mono Mac 1) cells (kindly provided by Dr. M. Tremblay, University of Laval) were cultured in RPMI, 10% FCS, 10 IU/ml penicillin, 10 IU/ml streptomycin and differentiated with LPS (10 ng/ml) for 72 hr (Genois *et al*, 2000) and subsequently infected with HIV-1 SF162 $10^4 TCID_{50}$/ml in triplicate. HIV-1 infected and uninfected cells were harvested and days 3 and 6 post-infection from which RNA was extracted.

Luciferase assay
U373/THP-1 cells were lysed in 250 μl 1x Cell Lysis Culture Reagent (Promega, Madison, USA). After a 15 min incubation period at RT, the lysate was harvested and centrifuged for 5 min at maximum speed. Supernatant was mixed with Luciferase Assay substrate (Promega) and, immediately, light emission was measured in a luminometer (Biocounter M2500, Lumac) at 562 nm.

RT-PCR
After 48 hours, transfected cells were homogenized and lysed in TRIzol (Life Technologies, Gaithersburg, MD) according to the manufacturer's guidelines. Total RNA was isolated and dissolved in diethylpyrocarbonate (DEPC)-treated water. RNA was treated with DNAase from which 1 μg of RNA was used for the synthesis of cDNA and subsequent PCR reactions were performed, as described previously (Boven *et al*, 1999). For semi-quantification, each primer pair was tested at different cycle numbers to determine the linear range. GAPDH mRNA levels were measured at 23 cycles, whereas cDNA was subjected to 25 cycles to be in the linear range for detection of MCP-1 and IL-1ß, and 35 cycles for Tat. Aliquots of 5 μl of the biotinylated PCR product were semi-quantitatively analyzed using a fluorescent digoxigenin detection ELISA kit (Boehringer Mannheim) according to manufacturer's protocol as described previously (Boven *et al*, 1999). All data were normalized against GAPDH mRNA levels. For confirmation of the microarray studies, real-time RT-PCR was performed using cDNA from 18 F(-83)A- and 12 L(-9)B-transfected cells using an established protocol (Boven *et al*, 2003). All sequences of the primers and probes are shown in Table 1.

cDNA microarray
HIV-1 Tat HAD (18 F(-83)A) and ND (12 L(-9)B) stably transfected U-937 cells were used to prepare a RNA pool. A previously reported array was used containing 19.2 K human genes spotted on a glass slide (Liu *et al*, 2004). Total RNA (10 μg) was added to 8 μl of 5$\times$ first strand buffer, to which a buffered solution containing SuperScript II reverse transcriptase (Life Technologies Inc., Burlington, ON) was added to each RNA sample and reverse transcription was performed. Cy3 and Cy5 dyes (Pharmacia, Piscataway, New Jersey) were resuspended in 18 μl DMSO and 0.625 μl of dye added to each 4.5 μl aliquot of cDNA. Coupling was performed in darkness at 45°C for 1 hr. Cy3 and Cy5 reactions were then combined for each set of reference and test samples. Arrays were prehybridized for 1 hour at 37°C with yeast tRNA,

Table 1 Sequences of oligonucleotide primers and probes in reverse transcriptase polymerase chain reaction

Target (product size)		*Sequence 5′-3′*
GAPDH	Sense	CCATGGAGAAGGCTGGGG
(195 bp)	Antisense	CAAAGTTGTCATGGATGACC
	Probe	CTGCACCACCAACTGCTTAGC
IL-1β	Sense	GCATCCAGCTACGAATCTCCGACC
(328 bp)	Antisense	CACTTGTTGCTCCATATCCTGTCCC
	Probe	GGACCAGACATCACCAAGCTTTTTGCTG
MCP-1	Sense	GCGAGCTATAGAAGAATCACC
(230 bp)	Antisense	ATAAAACAGGGTGTCTGGGG
	Probe	GACAAGCAAACCCAAACTCC
HIV *tat/rev*	Sense	GGCTTAGGCATCTCCTATGGC
(123 bp)	Antisense	TGTCGGGTCCCCTCGTTGCTGG
	Probe	CTTTGATAGAGAAACTTGATGAGTCTG
P2RX1 (187bp)	Sense	GTGCCCCGTGGAGGTGGATGA
	Antisense	CAGGGGGTGCAGGGTCTTGTGAA
BRE (164 bp)	Sense	TGACTCCTGGGCCCAACTGTGA
	Antisense	ATTCTGCAAAGCTGAGGGGTCTGG
HS3ST3B1 (89 bp)	Sense	CGCCGCCGGTGAGGAGGAAG
	Antisense	GCAGGAGCCGGCGCACGAGTA
TARBP1 (125 bp)	Sense	GCACGGAGCAGGGAATG
	Antisense	CTGAAAGGCGTGGAAGGAT
ADNP (142 bp)	Sense	CCCTCTCGGCTTAATCAGTCTCCA
	Antisense	CAGGCTCTTCAGGCTTCTCTTCAA
PIP5K2B (77 bp)	Sense	CGCAGCGCCCCCATCAACAGT
	Antisense	ACAAAGCGCCGGTCGTAGGTGGTG
IFITM-1 (256 bp)	Sense	TGAGCACCGTCCCAGCATCC
	Antisense	AAGCCCAGACAGCACCAGTTCAAG
HIST1H2BG (77 bp)	Sense	CGCAAGCGCAGTCGTAAG
	Antisense	ATGCCAGTATCGGGGTGAAC
UXS1 (220 bp)	Sense	AGGAAGGCGTGGAAGTGCGAGTGG
	Antisense	ACCGGGCTGCTGACGTTGCTGTTC
F2 (211 bp)	Sense	GTGTGGGGCCAGCCTCATCAGT
	Antisense	GTCCAGGTTCTCCCGCCAGTTGTA
PDCD7 (145 bp)	Sense	CCGCTGGAGGGTGAAGTGTG
	Antisense	CTCCAAAGCCCGTAGAATGTCC

salmon sperm DNA and 10% BSA in human hybridization chambers. Probes were resuspended and then hybridized on glass arrays overnight at 37°C in hybridization chambers. Slides were dried by centrifugation before scanning using the GenePix 4000 scanner (Axon Instruments Inc., Union City, CA). Data were analyzed using QuantArray image analysis software (GSI Lumonics, Ottawa, ON). Cy3 and Cy5 channels were normalized by subarray intensity and ratios were calculated as test sample divided by reference sample on each slide. Gene Traffic microarray data analysis software (version 3.2, Iobion Informatics) was used to evaluate hybridization data. Genes exhibiting a mean $\log_2$ ratio ≥ 1 or ≤ -1 in transcript levels for HAD (18 F(-83)A) to ND (12 L(-9)B) in transfected cells and a coefficient of variation $\leq 50\%$ were considered for further analysis. Each gene had at least 4 valid hybridization spots across 2 hybridizations.

Human brain tissue samples
Brain tissue (frontal white matter) was collected at autopsy from HIV/AIDS patients and frozen at −80°C from which RNA was extracted, as described previously (Boven *et al*, 2003; Tsutsui *et al*, 2004). Patients were stratified into demented (HAD) and nondemented (ND) groups based on standard neurological and neuropsychological evaluations with matching ages and levels of immunosuppression (Power *et al*, 1993; Power *et al*, 1998).

Statistical analysis
Statistical analyses were performed using GraphPad InStat version 3.0 (GraphPad Software, San Diego California USA, www.graphpad.com) for both parametric and nonparametric comparisons. p values of less than 0.05 were considered significant.

Results

Brain-derived HIV-1 Tat clones exhibit variable transactivation of the HIV-1 LTR
Earlier studies have shown that HIV-1 Tat proteins may have differing actions on HIV-1 LTR transactivation depending on the Tat sequence (Roof *et al*, 2002). To examine the effects of different brain-derived *tat* sequences (Figure 1A), which were representative of HIV/AIDS patients with or without HAD (Bratanich *et al*, 1998), we co-transfected U373 or THP-1 cells with prototypic HAD (n = 3) or ND (n = 3) Tat clones and a HIV LTR construct, with subsequent measurement of HIV LTR transactivation by luciferase assay. Clones 12 L(-9)C, 26 D(-156)E, and 26 C(-169)A were derived from brain tissue of non-demented (ND) AIDS patients, while clones 18 F(-83)A, 18 F(-83)C, and 25 E(-106)A were derived from individuals with HAD (Bratanich *et al*, 1998). In contrast to clones derived from ND individuals, Tat clones from individuals with HAD showed minimal transactivation of the HIV LTR ($p < 0.05$) (Figure 1B). Although there were no significant differences between THP-1 and U373 cells in terms of LTR activation, U373 cells generally permitted greater LTR activation, perhaps due to differing transfection efficiencies. To investigate whether differences in LTR transactivation were caused by differences in transcript levels, *tat* mRNA sequence abundance was measured in transfected U373 and THP-1 cells (Figure 1C). All clones were expressed (Figure 1C) although no correlation between *tat* expression levels and LTR transactivation was observed, despite some variation in *tat* transcript levels. Thus, these studies indicated that there was variability in LTR transactivation by different *tat* sequences despite comparable levels of gene expression by each of the *tat*-encoding vectors.

Transactivation variation among different tat clones for other LTR constructs
The Tat protein is known to bind different transcription factors, including SP-1, TBP-1, TAK and TAP (Gatignol *et al*, 1996), which influence Tat-mediated transcription (Kelly *et al*, 1999). As mutations in Tat might alter binding of host transcription factors which in turn transactivate the LTR, HIV-1 LTR constructs containing variable binding domains (Figure 2A) were used in co-transfections with the above Tat clones. Studies of LTR transactivation revealed that deletion or truncation of different transcription sites resulted in an overall reduction in transactivation for all Tat clones depending on the individual LTR clone, compared to the full length LTR clone (Figure 1). Indeed, the shortest LTR construct (HIV-1 LTR2), containing only one SP1 site, mediated the lowest transcription levels. However, all of the ND Tat clones transactivated the different LTRs while the HAD Tat clones again displayed minimal transactivation of each LTR construct (Figure 2B), similar to the full length LTR clone (Figure 1B). These findings underscored the marked effects of Tat molecular diversity on LTR transactivation.

Select Tat-derived peptides inhibit LTR transactivation
As the Tat amino acids 20–40 have been identified as a domain, which is crucial for viral replication and NF-κB activation (Huigen *et al*, 2004), we investigated the involvement of this domain in LTR transactivation. Peptides derived from prototypic ND (12 L(-9)B) and HAD (18 F(-83)A) Tat sequences were prepared (Figure 3A). To determine if these peptides inhibited LTR transactivation through competition for the Tat-binding site(s) in the LTR, co-transfections were performed with the 12 L(-9)B clone and the full length pHIV-LTR in the presence of two different 112 L(-9)B derived peptides. The 24–38 peptide derived from 12 L(-9)B inhibited LTR transactivation in a dose-dependent pattern (Figure 3B), by 60% for the highest peptide concentration in contrast to the homologous peptide derived from 18 F(-83)A ($p < 0.01$), while the other two peptides derived from 12 L(-9)B

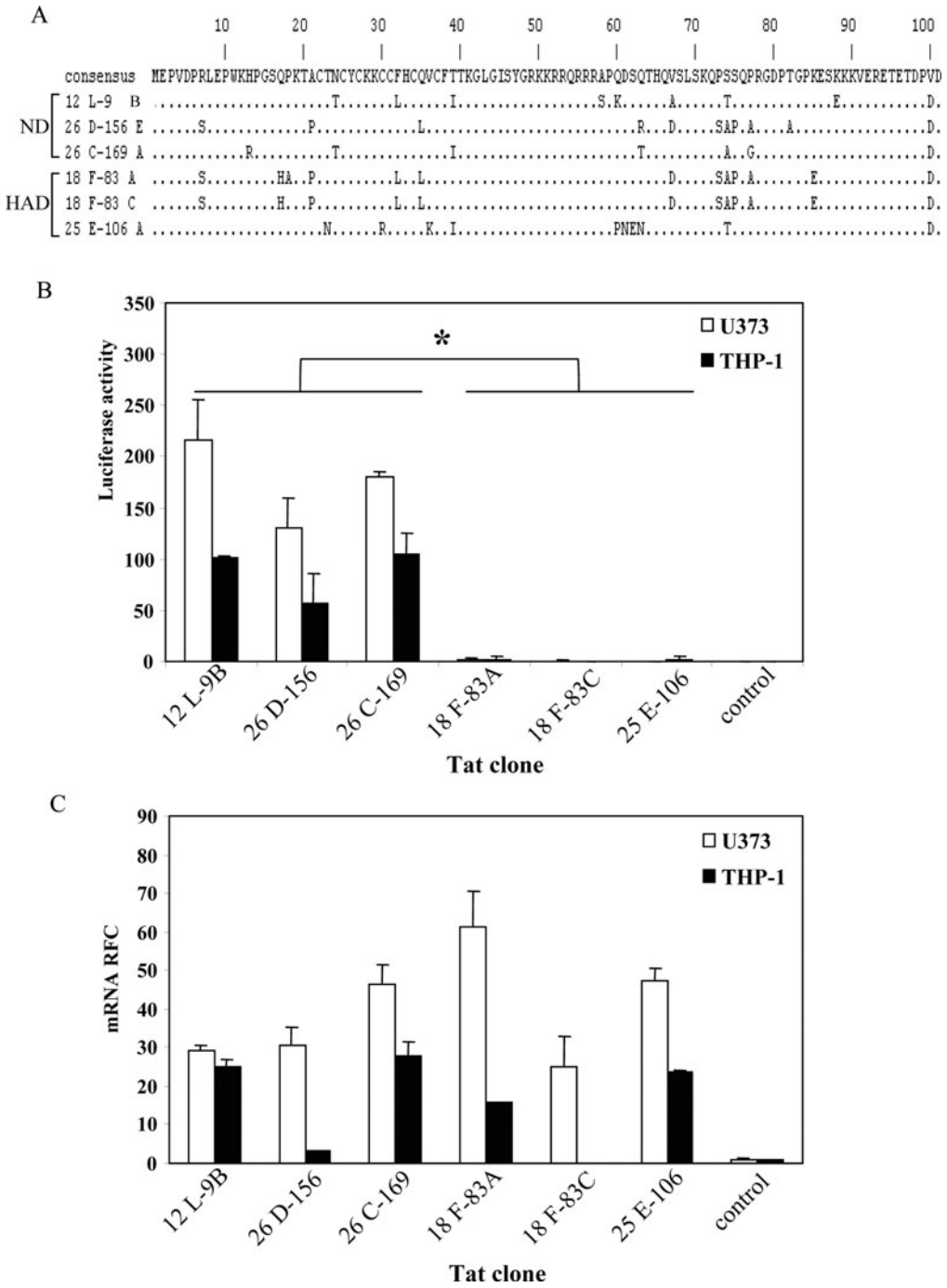

Figure 1 HIV-1 LTR transactivation mediated by brain-derived Tat clones. (A) Brain-derived clones 12 L(-9)B, 26 D(-156)E, and 26 C(-169)A were derived from non-demented HIV/AIDS patients (ND) while clones 18 F(-83)A, 18 F(-83)C, and 25 E(-106)A were derived from individuals with HIV-associated dementia (HAD). (B) Luciferase activity (mean ± SEM) in U373 and THP-1 cells was measured 48 hours after transfection, showing that ND *tat* clones efficiently transactivated the HIV-1 LTR. (C) mRNA relative fold change (RFC) (± SEM) in *tatclones* mRNA levels were measured in cells transfected with the different clones demonstrating that *tat* mRNA levels did not differ amongst the various clones. Controls represent mock transfections performed in triplicate. ($^*p < 0.05$).

and 18 F(-83)A, respectively, did not demonstrate any inhibition of LTR transactivation. These studies suggested that the 12 L(-9)B-derived peptide containing residues 24–38 inhibited LTR activation in a sequence-specific manner unlike the matching 18 F(-83)A-derived peptide.

All brain-derived Tat clones induce host immune responses

Although Tat is chiefly recognized for its ability to transactivate the LTR, it also exhibits direct effects on host gene expression levels, including induction of cytokines and chemokines (Nath, 2002). To

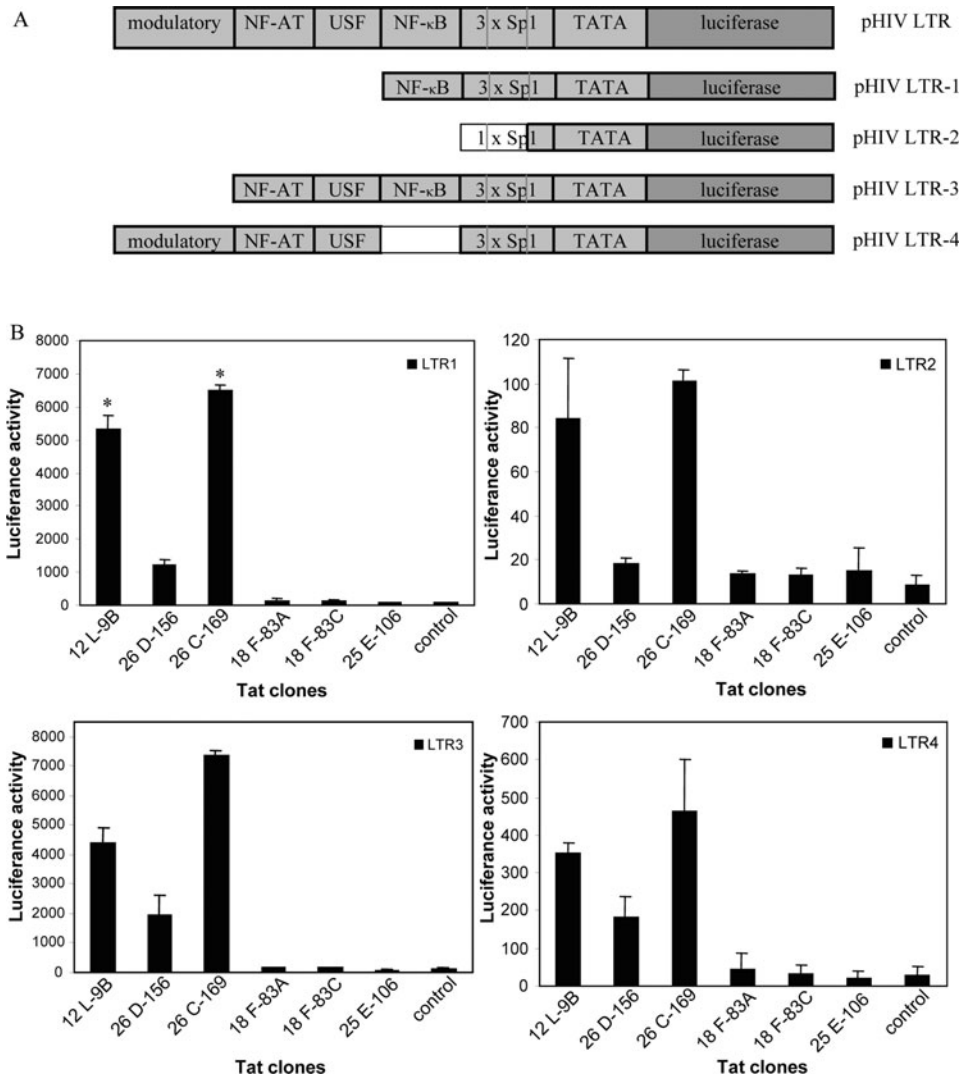

Figure 2 Activation of different LTR constructs by brain-derived Tat clones. After co-transfection of astrocytic (U373) cells with different HIV-1 *tat* clones and LTR constructs (A), mean LTR activity (± SEM) was measured (B). LTR transactivation was dependent on both *tat* and LTR sequences although the HAD *tat* clones consistently showed less LTR transactivation, regardless of the LTR sequence ($^*p < 0.05$).

investigate whether the different Tat clones induced an innate immune response, we examined IL-1ß and MCP-1 mRNA levels following transfection of relevant cell types (Figure 4). In U373 cells, both MCP-1 (Figure 4A) and IL-1ß (Figure 4B) were induced by all *tat* clones as compared to non-transfected U373 cells. Although all *tat* clones induced IL-1β (Figure 4B) in THP-1 cells, they induced low levels of MCP-1 in the same cell type (Figure 4A). However, no significant differences were observed among the different HAD and ND *tat* clones in terms of host gene induction and moreover, the ability of the individual *tat* clones to induce host gene expression was independent of their ability to transactivate the LTR.

Genomic profiling of Tat-induced host gene induction

Although differences in host neuroimmune gene induction were not observed for the above HAD- and ND-derived *tat* sequences, we examined the differential effects of two prototypic *tat* sequences by large scale genomic analysis. Using monocytoid (U-937) cells that were stably transfected with the 18 F(-83)A (HAD) or 12 L(-9)B (ND) brain-derived clones and known to express Tat proteins (Johnston *et al*, 2001c), we performed cDNA microarray studies using an established 19.2 K gene chip (Liu *et al*, 2004) to compare the relative effects of Tat molecular diversity. Microarray data analysis across two hybridizations showed up-regulation of 14 genes in 18 F(-83)A-compared to 12 L(-9)B-transfected cells (mean $\log_2$ ratio ≥ 1) (Figure 5A) including ADNP (activity-dependent neuroprotector), PIP5K2B (phosphatidylinositol-4-phosphate 5-kinase type II beta), IFITM1 (interferon induced transmembrane protein 1), HS3ST3B1 (heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1), KIAA0329 (KIAA0329), F2 (thrombin), TARBP1 (TAR-HIV RNA

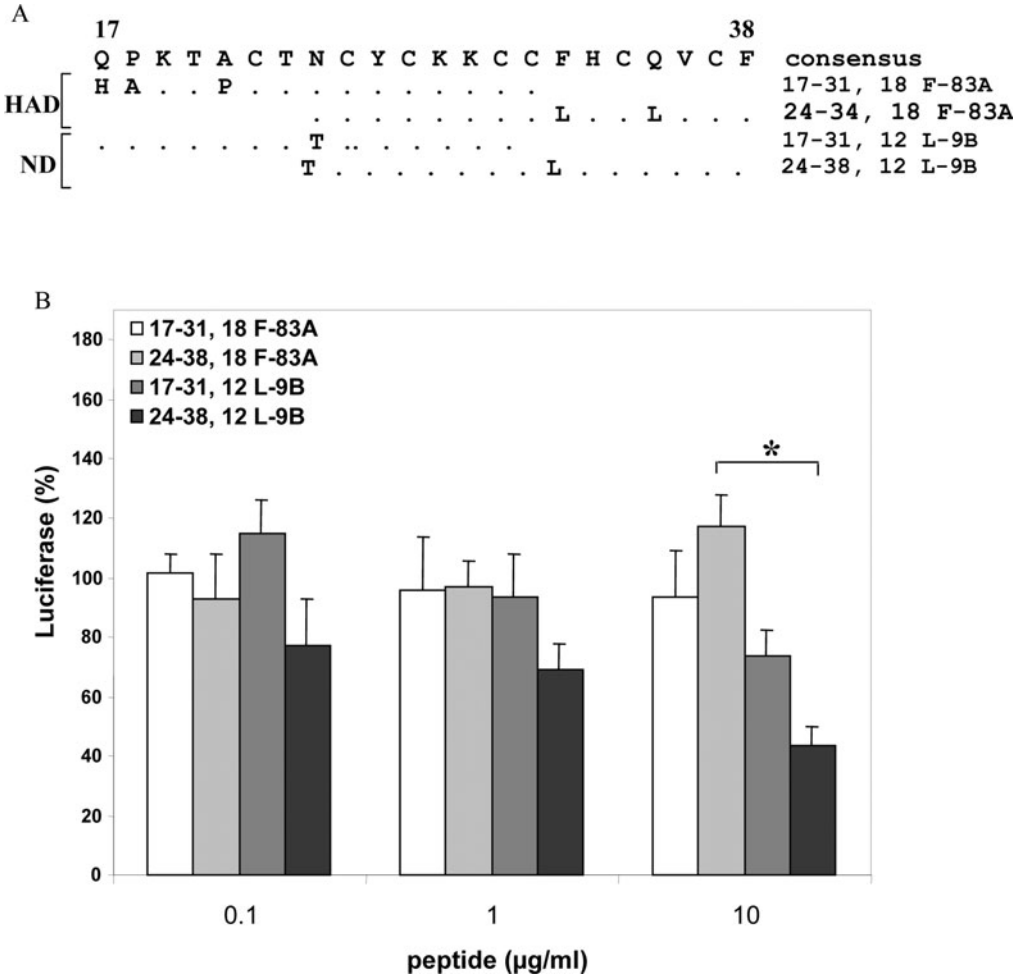

Figure 3 Inhibition of LTR activation by peptide 24-38, 12 L(-9)B. (A) Different Tat-derived peptides were applied to astrocytic (U373) cells transfected with a ND (12 L(-9)B) *tat* clone. (B) Mean LTR activity (± SEM), expressed relative to cells co-transfected with the 12 L(-9)B plasmid in the absence of the 12 L(-9)B-derived peptide, was measured revealing that only the Tat 12 LB-derived peptide (AA 24-38) inhibited LTR transactivation, which was significantly reduced in a concentration-dependent manner ($^{*}p < 0.05$).

binding protein 1), BRE (brain and reproductive organ-expressed), P2RX1 (purinergic receptor P2X), CPOX (coproporphyrinogen oxidase), HIST1H2BG (histone 1 H2bg), UXS1 (UDP-glucuronate decarboxylase 1), PKD2 (polycystic kidney disease 2) and LIN7A (lin-7 homolog A). Indeed, only 3 genes (PDCD7, FLJ39739 and GMPS) exhibited relative down-regulation (mean $\log_2$ ratio ≤ 1) following comparison of 18 F(-83)A versus 12 L(-9)B induction (Figure 5A). To confirm the differential gene expression revealed by the microarray hybridizations, we also examined the mRNA levels of these genes in 18 F(-83)A- or 12 L(-9)B-transfected U-937 cells using real-time RT-PCR. Of the fourteen genes upregulated in the microarray analysis, 2 genes were also found to be up-regulated significantly by RT-PCR analysis, including HS3ST3B1 and IFITM1 (Figure 5B). Comparison of the relative abundance of the latter two genes in brains from ND and HAD patients revealed a significant increase in HAD patients for HS3ST3B1, underscoring the specificity of gene induction mediated by HIV-1 Tat (Figure 5C). However, among those genes down regulated on the microarray analysis, transcript levels of the pro-apoptotic gene, PDCD7, was also significantly suppressed in the 18 F(-83)A-transfected cells (Figure 5B). In fact, infection of differentiated monocytoid (MonoMac-1) cells with the neurotropic HIV-1 strain, SF162, showed a significant suppression of PDCD7 transcripts at day 3 post infection, which was more evident at day 6 post-infection (Figure 5D). These findings emphasize the utility of using large scale genomic profiling to discern the actions of individual HIV-1 genes and their potential effects on pathogenesis.

Discussion

The present study highlights the diverse effects of the HIV-1 Tat protein in terms of its capacity to influence viral replication, host immune response and its potential to influence neuropathogenesis in

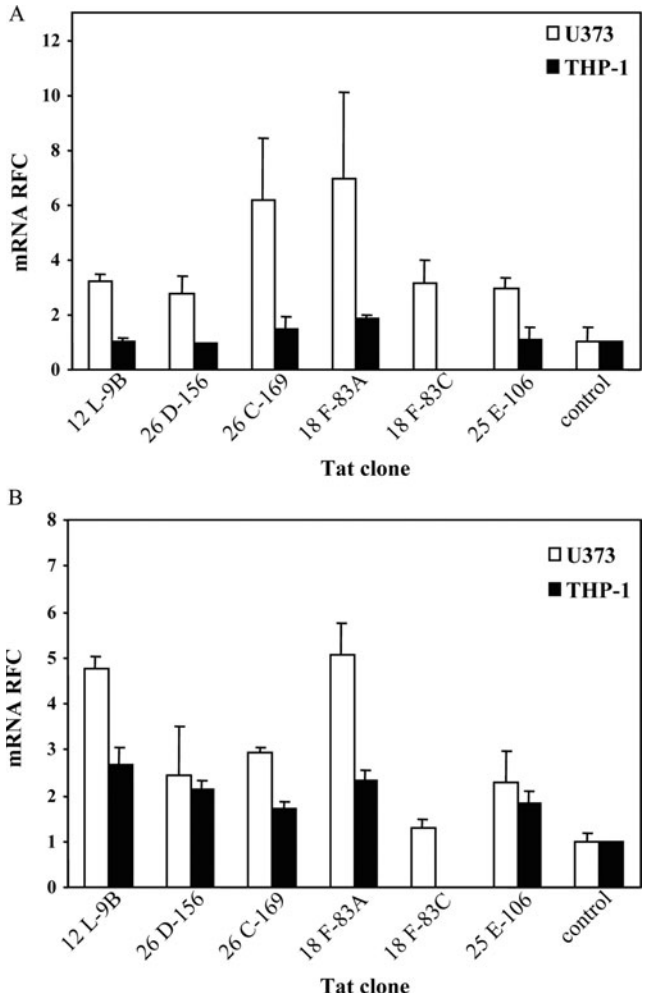

Figure 4 MCP-1 and IL-1β mRNA levels are increased in Tat-transfected U373 and THP-1 cells. U373 and THP-1 cells were transfected with the different brain-derived HIV-1 Tat clones and after 48 hours RNA was isolated and semi-quantitative RT-PCR was performed for MCP-1 (A) and IL-1β (B). All Tat-transfected cells exhibited induced MCP-1 and IL-1ß mRNA expression although MCP-1 levels were low in THP-1 cells.

a sequence-dependent manner. Herein, we show that while brain-derived Tat sequences can modulate LTR activity depending on the domain, the specific sequence and clinical group from which the sequence was derived, they also affect host responses depending on the cell type and host gene of interest. Genomic profiling disclosed altered expression for several genes, including an enzyme mediating heparan sulphate synthesis and a pro-apoptotic molecule (PDCD7), affected by Tat in a sequence-dependent manner. Both genes had not been previously recognized to participate in HIV-1 pathogenesis. Indeed, these latter findings were verified by subsequent analysis of relevant brain tissues or HIV-infected monocytoid cells. Thus, the HIV-1 Tat exerts a wide diversity of effects contributing to neuropathogenesis, representing an important target for future interventions in the treatment of HIV-1 related neurological disorders.

HIV-1 Tat has been implicated in a range of HIV/AIDS-related disorders and most importantly, is widely assumed to play an important role in HAD (reviewed in (Peruzzi *et al*, 2002)). Since it was reported that HIV-1 *tat* sequences demonstrate substantial molecular diversity in the brain (Bratanich *et al*, 1998), in this study we sought to assess the biological effects of this sequence variation. In particular, we were interested in differences in LTR transactivation capacity, since many of the mutations were located in the domain that is associated with viral replication, amino acids 21–40 (Boykins *et al*, 1999). Indeed, we observed substantial differences in LTR activation by multiple HIV-1 *tat* clones, all of which were isolated from brain tissue of HIV/AIDS patients with or without HIV-associated dementia. In fact, HIV-1 *tat* clones isolated from individuals with HAD failed to transactivate the HIV-1 LTR to any extent while *tat* clones derived from ND individuals (without HAD) were uniformly able to activate the HIV-1 LTR. Decreased binding of Tat to one or more of transcription factor(s) may cause differences in LTR transactivation (Bannwarth and Gatignol, 2005). Since differences in Tat-mediated transactivation were maintained using a LTR construct with only a single SP1 site, mutations in Tat may result in an aberrant interaction with SP1. Indeed, earlier studies suggest that Tat may not directly interact with SP1 (Loregian *et al*, 2003) although these interactions remain to be fully elucidated (Rossi *et al*, 2006). Nonetheless, analyses of the present HAD *tat* clone (18 F(-83)A) in other assays of LTR activity revealed that it was able to transactivate LTR derived sequences from different HIV-1 clades, albeit also at low levels (Silva *et al*, 2003). In the current study only six full length Tat clones were investigated although there was an association between select Tat domains, low LTR transactivation and the presence of HAD. This finding is in agreement with previous data that show there is not a definitive correlation between brain viral load and the development of HAD (reviewed in (Gonzalez-Scarano and Martin-Garcia, 2005)). It is also plausible that during advanced HIV-1 infection of the brain, mutations in individual genes might lead to a loss of (neurovirulent) function and "retirement" of the gene as an evolutionary strategy for viral persistence (van Marle and Power, 2005). Important issues to address in the future include the role of demonstrated cyclin T1's interaction with Tat in neural cells (Darbinian *et al*, 2001) and a possible role for perturbed Tat acetylation in HAD. Other studies suggest that HIV-1 neurovirulence and viral replication are not closely coupled (Zhang *et al*, 2003b) and therefore, mutated Tat protein might contribute to the HAD pathogenesis through various disease mechanisms with limited viral replication. There might also be specific requirements in terms of HIV-1 LTR sequence for the present HAD-derived *tat* clones to be functional. Importantly, recent studies suggest that

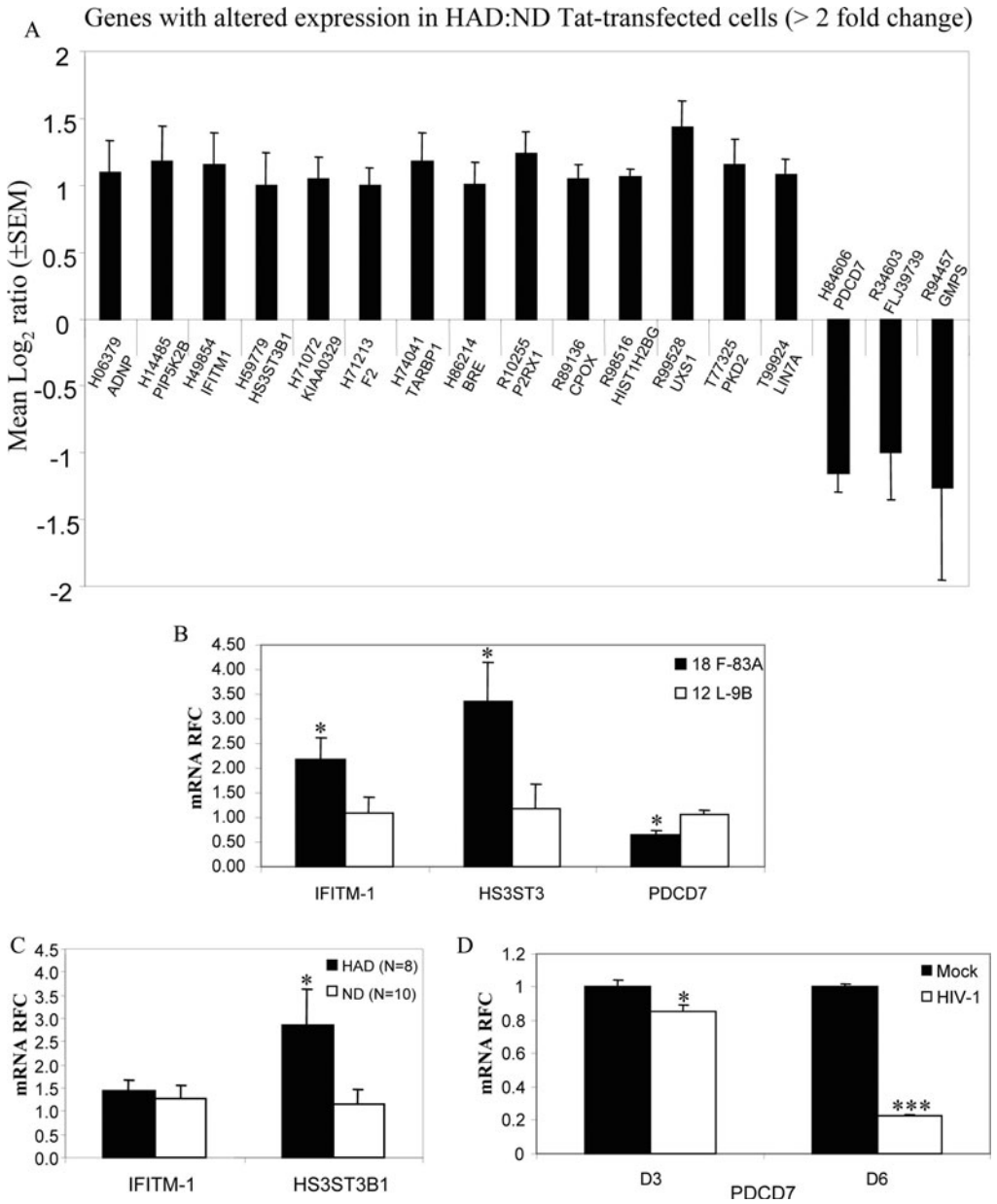

Figure 5 HAD and ND Tat clones exhibit differential gene expression. (A) Transcription profiling by cDNA microarray analysis showed a relative up-regulation of 14 genes in 18 F(-83)A-compared to 12 L(-9)B-transfected monocytoid (U-937) cells, assuming a mean $\log_2$ ratio ≥ 1 to be a meaningful increase in gene expression. (B) Real-time RT-PCR analysis confirmed the up-regulation of IFITM-1 and HS3ST3B1 and suppression of PDCD7 transcript levels in the same cells. (C) HAD brains also showed induction of HS3ST3B1 transcripts compared to ND patients' brain samples. (D) Infection of differentiated monocytoid cells with HIV-1 SF162 showed down regulation of PDCD7 transcript abundance at days 3 and 6 post-infection compared to mock-infected cells. ($^{*}p < 0.05$, $^{***}p < 0.001$).

LTR sequences from HAD patients display distinct transcription factor binding properties compared to ND-derived *tat* sequences (Burdo *et al*, 2004).

Mutations in the 17–38 AA domain of the HIV-1 Tat protein might be involved in the Tat-mediated LTR transactivation, based on the present findings. Assuming the peptides were able to bind to the LTR, a decrease in Tat-mediated LTR transactivation was anticipated, since the current peptides lack other necessary domains to induce activation. Notably, the mutations at position 35, glutamine to a leucine, and at position 24, threonine to asparagine appeared to have the principal effects on LTR transactivation. The 18 F(-83)A-derived peptide (24-38) likely competed with the functional 12 L(-9)B Tat-encoded protein because it did not inhibit Tat-mediated LTR transactivation. Of interest, these mutations were frequently observed in brain-derived Tat sequences from individuals with HAD compared to those individuals without HAD (Bratanich *et al*, 1998). Herein, we

observed diminished HIV-1 LTR transactivation for one of the clones that was isolated from a nondemented individual (26 D(-156)E). Interestingly, this latter clone also demonstrated asparagine on position 24 and a leucine on position 35. One of the clones that was derived from an individual with HAD (25 E(-106)A) did not demonstrate the leucine at position 35, although it did have a mutation of an adjacent amino acid. These findings emphasize the complexity of Tat's effects on LTR transactivation, which are dependent on both LTR and Tat sequence diversity.

Although the present *tat* clones demonstrate differences in LTR transactivation, Tat-mediated induction of the inflammatory genes, MCP-1 and IL-1ß, was not substantially different among the different *tat* clones. This observation suggests that the mechanism that leads to increased expression of these genes is different from the mechanism resulting in LTR transactivation. These data are in support of a study, which postulates that Tat functions through two separate pathways. One mechanism involves the Tat/TAR interaction and the other through a TAR-independent pathway, which results in activation of cytosolic NF-κB and subsequent activation of NF-κB-mediated genes, such as TNF-α, MCP-1, and IL-1ß (Biswas *et al*, 1995). Nonetheless, the present microarray revealed that 14 genes exhibited enhanced expression in 18 F(-83)A- compared to 12 L(-9)B-transfected monocytoid cells. Importantly, supernatants from these same stably tranfected cells were found previously to be differentially cytotoxic to human and murine neurons (Johnston *et al*, 2001b). The underlying mechanism for this latter observation was related to MMP-2 production, which was cytotoxic to neurons (Zhang *et al*, 2003a). However, the present results revealed that other genes were also differentially upregulated. In particular, RT-PCR confirmation established the enhanced expression of the enzyme, HS3ST3B1, involved in the synthesis of heparan sulphate, which could have direct pathogenic effects in terms of enhancing HIV-1 entry. Given that HIV-1 uses heparan sulphate for cellular entry (Patel *et al*, 1993), it is conceivable that the present finding might reflect a cooperative effect in which Tat enhances infection of adjacent cells through its effects on HS3ST3B1. Indeed, a recent study (Marchio *et al*, 2005) suggests that Tat adherence to heparan sulphate on permissive cells may modulate HIV-1 infection. Similarly, a gene implicated in apoptosis, PDCD7 (Park *et al*, 1999), was suppressed in the microarray analysis and also in HIV-1 infected monocytoid cells, which might reflect a distinct protective effect mediated by Tat in HIV-infected monocytoid cells in the brain. By preventing the death of HIV-infected cells through suppression of apoptosis, HIV-1 persistence in the brains might be augmented. These effects as well as the other genes activated by the HAD *tat* sequence will require further analysis in the future.

References

Aldovini A, Feinberg MB (1990). *Techniques in HIV research*. Stockton Press: New York.

Andreoni M (2004). Viral phenotype and fitness. *New Microbiol* **27:** 71–76.

Bannwarth S, Gatignol A (2005). HIV-1 TAR RNA: the target of molecular interactions between the virus and its host. *Curr HIV Res* **3:** 61–71.

Biswas DK, Salas TR, Wang F, Ahlers CM, Dezube BJ, Pardee AB (1995). A Tat-induced auto-up-regulatory loop for superactivation of the human immunodeficiency virus type 1 promoter. *J Virol* **69:** 7437–7444.

Boven LA, Gomes L, Hery C, Gray F, Verhoef J, Portegies P, Tardieu M, Nottet HS (1999). Increased peroxynitrite activity in AIDS dementia complex: implications for the neuropathogenesis of HIV-1 infection. *J Immunol* **162:** 4319–4327.

Boven LA, Vergnolle N, Henry SD, Silva C, Imai Y, Holden J, Warren K, Hollenberg MD, Power C (2003). Up-regulation of proteinase-activated receptor 1 expression in astrocytes during HIV encephalitis. *J Immunol* **170:** 2638–2646.

Boykins RA, Mahieux R, Shankavaram UT, Gho YS, Lee SF, Hewlett IK, Wahl LM, Kleinman HK, Brady JN, Yamada KM, Dhawan S (1999). Cutting edge: a short polypeptide domain of HIV-1-Tat protein mediates pathogenesis. *J Immunol* **163:** 15–20.

Brack-Werner R (1999). Astrocytes: HIV cellular reservoirs and important participants in neuropathogenesis. *Aids* **13:** 1–22.

Bratanich AC, Liu C, McArthur JC, Fudyk T, Glass JD, Mittoo S, Klassen GA, Power C (1998). Brain-derived HIV-1 tat sequences from AIDS patients with dementia show increased molecular heterogeneity. *J Neurovirol* **4:** 387–393.

Burdo TH, Nonnemacher M, Irish BP, Choi CH, Krebs FC, Gartner S, Wigdahl B (2004). High-affinity interaction between HIV-1 Vpr and specific sequences that span the C/EBP and adjacent NF-kappaB sites within the HIV-1 LTR correlate with HIV-1-associated dementia. *DNA Cell Biol* **23:** 261–269.

Chen P, Mayne M, Power C, Nath A (1997). The Tat protein of HIV-1 induces tumor necrosis factor-alpha production. Implications for HIV-1-associated neurological diseases. *J Biol Chem* **272:** 22385–22388.

Conant K, Garzino-Demo A, Nath A, McArthur JC, Halliday W, Power C, Gallo RC, Major EO (1998). Induction of monocyte chemoattractant protein-1 in HIV-1 Tat-stimulated astrocytes and elevation in AIDS dementia. *Proc Natl Acad Sci USA* **95:** 3117–3121.

Conant K, Ma M, Nath A, Major EO (1996). Extracellular human immunodeficiency virus type 1 Tat protein is associated with an increase in both NF-kappa B binding and protein kinase C activity in primary human astrocytes. *J Virol* **70:** 1384–1389.

Darbinian N, Sawaya BE, Khalili K, Jaffe N, Wortman B, Giordano A, Amini S (2001). Functional interaction between cyclin T1/cdk9 and Puralpha determines the level

of TNFalpha promoter activation by Tat in glial cells. *J Neuroimmunol* **121:** 3–11.

Dayton AI, Sodroski JG, Rosen CA, Goh WC, Haseltine WA (1986). The trans-activator gene of the human T cell lymphotropic virus type III is required for replication. *Cell* **44:** 941–947.

Gatignol A, Duarte M, Daviet L, Chang YN, Jeang KT (1996). Sequential steps in Tat trans-activation of HIV-1 mediated through cellular DNA, RNA, and protein binding factors. *Gene Expr* **5:** 217–228.

Genois N, Robichaud GA, Tremblay MJ (2000). Mono Mac 1: a new in vitro model system to study HIV-1 infection in human cells of the mononuclear phagocyte series. *J Leukoc Biol* **68:** 854–864.

Gonzalez-Scarano F, Martin-Garcia J (2005). The neuropathogenesis of AIDS. *Nat Rev Immunol* **5:** 69–81.

Gorman CM, Moffat LF, Howard BH (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol Cell Biol* **2:** 1044–1051.

Hofman FM, Dohadwala MM, Wright AD, Hinton DR, Walker SM (1994). Exogenous tat protein activates central nervous system-derived endothelial cells. *J Neuroimmunol* **54:** 19–28.

Huigen MC, Kamp W, Nottet HS (2004). Multiple effects of HIV-1 trans-activator protein on the pathogenesis of HIV-1 infection. *Eur J Clin Invest* **34:** 57–66.

Johnston JB, Silva C, Gonzalez G, Holden J, Warren KG, Metz LM, Power C (2001a). Diminished adenosine A1 receptor expression on macrophages in brain and blood of patients with multiple sclerosis. *Ann Neurol* **49:** 650–658.

Johnston JB, Silva C, Holden J, Warren KG, Clark AW, Power C (2001b). Monocyte activation and differentiation augment human endogenous retrovirus expression: implications for inflammatory brain diseases. *Ann Neurol* **50:** 434–442.

Johnston JB, Zhang K, Silva C, Shalinsky DR, Conant K, Ni W, Corbett D, Yong VW, Power C (2001c). HIV-1 Tat neurotoxicity is prevented by matrix metalloproteinase inhibitors. *Ann Neurol* **49:** 230–241.

Kelly GD, Morris CB, Offermann MK (1999). Lack of responsiveness of a nuclear factor-kappaB-regulated promoter to transactivation by human immunodeficiency virus 1 Tat in HeLa cells. *Virology* **263:** 128–138.

Lassen K, Han Y, Zhou Y, Siliciano J, Siliciano RF (2004). The multifactorial nature of HIV-1 latency. *Trends Mol Med* **10:** 525–531.

Liu J, Blackhall F, Seiden-Long I, Jurisica I, Navab R, Liu N, Radulovich N, Wigle D, Sultan M, Hu J, Tsao MS, Johnston MR (2004). Modeling of lung cancer by an orthotopically growing H460SM variant cell line reveals novel candidate genes for systemic metastasis. *Oncogene* **23:** 6316–6324.

Loregian A, Bortolozzo K, Boso S, Sapino B, Betti M, Biasolo MA, Caputo A, Palu G (2003). The Sp1 transcription factor does not directly interact with the HIV-1 Tat protein. *J Cell Physiol* **196:** 251–257.

Marchio S, Alfano M, Primo L, Gramaglia D, Butini L, Gennero L, De Vivo E, Arap W, Giacca M, Pasqualini R, Bussolino F (2005). Cell surface-associated Tat modulates HIV-1 infection and spreading through a specific interaction with gp120 viral envelope protein. *Blood* **105:** 2802–2811.

Mayne M, Bratanich AC, Chen P, Rana F, Nath A, Power C (1998). HIV-1 tat molecular diversity and induction of TNF-alpha: implications for HIV-induced neurological disease. *Neuroimmunomodulation* **5:** 184–192.

Nabel G, Baltimore D (1987). An inducible transcription factor activates expression of human immunodeficiency virus in T cells. *Nature* **326:** 711–713.

Nath A (2002). Human immunodeficiency virus (HIV) proteins in neuropathogenesis of HIV dementia. *J Infect Dis* 186 Suppl **2:** S193–S198.

Nath A, Conant K, Chen P, Scott C, Major EO (1999). Transient exposure to HIV-1 Tat protein results in cytokine production in macrophages and astrocytes. A hit and run phenomenon. *J Biol Chem* **274:** 17098–17102.

Park EJ, Kim JH, Seong RH, Kim CG, Park SD, Hong SH (1999). Characterization of a novel mouse cDNA, ES18, involved in apoptotic cell death of T-cells. *Nucleic Acids Res* **27:** 1524–1530.

Patel M, Yanagishita M, Roderiquez G, Bou-Habib DC, Oravecz T, Hascall VC, Norcross MA (1993). Cell-surface heparan sulfate proteoglycan mediates HIV-1 infection of T-cell lines. *AIDS Res Hum Retroviruses* **9:** 167–174.

Peruzzi F, Gordon J, Darbinian N, Amini S (2002). Tat-induced deregulation of neuronal differentiation and survival by nerve growth factor pathway. *J Neurovirol* 8 Suppl **2:** 91–96.

Power C, Kong PA, Crawford TO, Wesselingh S, Glass JD, McArthur JC, Trapp BD (1993). Cerebral white matter changes in acquired immunodeficiency syndrome dementia: alterations of the blood-brain barrier. *Ann Neurol* **34:** 339–350.

Power C, McArthur JC, Nath A, Wehrly K, Mayne M, Nishio J, Langelier T, Johnson RT, Chesebro B (1998). Neuronal death induced by brain-derived human immunodeficiency virus type 1 envelope genes differs between demented and nondemented AIDS patients. *J Virol* **72:** 9045–9053.

Rappaport J, Joseph J, Croul S, Alexander G, Del Valle L, Amini S, Khalili K (1999). Molecular pathway involved in HIV-1-induced CNS pathology: role of viral regulatory protein, Tat. *J Leukoc Biol* **65:** 458–465.

Roof P, Ricci M, Genin P, Montano MA, Essex M, Wainberg MA, Gatignol A, Hiscott J (2002). Differential regulation of HIV-1 clade-specific B, C, and E long terminal repeats by NF-kappaB and the Tat transactivator. *Virology* **296:** 77–83.

Rossi A, Mukerjee R, Ferrante P, Khalili K, Amini S, Sawaya BE (2006). Human immunodeficiency virus type 1 Tat prevents dephosphorylation of Sp1 by TCF-4 in astrocytes. *J Gen Virol* **87:** 1613–1623.

Silva C, Zhang K, Tsutsui S, Holden JK, Gill MJ, Power C (2003). Growth hormone prevents human immunodeficiency virus-induced neuronal p53 expression. *Ann Neurol* **54:** 605–614.

Tsutsui S, Schnermann J, Noorbakhsh F, Henry S, Yong VW, Winston BW, Warren K, Power C (2004). A1 adenosine receptor upregulation and activation attenuates neuroinflammation and demyelination in a model of multiple sclerosis. *J Neurosci* **24:** 1521–1529.

van der Zee R, Anderton SM, Buskens CAF, Alonso de Velasco E, van Eden W (1994). Heat shock protein T cell epitopes as immunogenic carriers in subunit vaccines. In: *Twenty-third European peptide symposium*. Maya HLS, (ed): Leiden.

van Marle G, Power C (2005). Human immunodeficiency virus type 1 genetic diversity in the nervous system:

evolutionary epiphenomenon or disease determinant? *J Neurovirol* **11:** 107–128.

Wesselingh SL, Power C, Glass JD, Tyor WR, McArthur JC, Farber JM, Griffin JW, Griffin DE (1993). Intracerebral cytokine messenger RNA expression in acquired immunodeficiency syndrome dementia. *Ann Neurol* **33:** 576–582.

Zhang K, McQuibban GA, Silva C, Butler GS, Johnston JB, Holden J, Clark-Lewis I, Overall CM, Power C (2003a). HIV-induced metalloproteinase processing of the chemokine stromal cell derived factor-1 causes neurodegeneration. *Nat Neurosci* **6:** 1064–1071.

Zhang K, Rana F, Silva C, Ethier J, Wehrly K, Chesebro B, Power C (2003b). Human immunodeficiency virus type 1 envelope-mediated neuronal death: uncoupling of viral replication and neurotoxicity. *J Virol* **77:** 6899–6912.